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Full Length Research Paper

Leptospirosis research: Response of pathogenic spirochete to ultaviolet-A irradiation

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The effect of exposure to ultaviolet-A (UVA) radiation was studied on the pathogenic spirochetes *Leptospira interrogans* serovar Canicola for different time durations. Changes in cell growth and viability due to UVA exposure were determined by using the conventional microscopic agglutination test (MAT), dark-field microscopy and spectrophotometry measurements. Changes in antigens and protein expression in the cells were detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot. Decrease in cell growth and viability was found to be related to the exposure period, or was dose dependent. The growth decreased sharply at a very high rate for the first 24 h of exposure (112.3 J/cm^2); then it reached the minimum within about a 1d of exposure and leveled off for further treatment until the 7d exposure period. Immunoblot revealed the presence of 21 kDa antigenic protein in the unexposed cells, which disappeared after exposure to UVA for 24 h. SDS-PAGE analysis indicated the presence of a 76 kDa protein band in the cells exposed to UVA for 2 to 24 h. Exposure to UVA for more than 24 h decreased this protein, but the proteins of molecular mass between 56 and 70 kDa appeared. This work is the first step toward understanding the effects of UVA on leptospira bacteria. Further investigation of the mechanisms involved in UVA interaction with leptospira will eventually lead to development of new strategies to control or prevent leptospira in the environment.

Key words: Ultraviolet-A, pathogenic spirochete, leptospirosis, *Leptospira interrogans.*

INTRODUCTION

Leptospirosis (a zoonotic disease caused by pathogenic spirochetes belonging to the family Leptospiraceae, genus Leptospira) has become a public health problem throughout the world. More than 250 serovars of this bacterium have been reported, based on the microscopic agglutination test (MAT). The problem is more severe in humid tropical and subtropical countries such as Thailand (Watt et al., 2003), Nicaragua (Brandling-Bennett et al., 1996) and India (WHO, 2000) than in countries having a temperate climate. The incidence of disease ranges from approx 10-100 per 100,000 to 0.1-1 per 100,000 per year in the humid tropics and temperate climates (WHO, 2003). The disease is transmitted via indirect contact with contaminated water and soil (Levett, 2001) or direct exposure to infected animals and their products (Douglin et al., 1997; Tangkanakul et al., 2000), mainly urine. Both wild and domestic animals, such as rodents, heifers and canines, are its natural hosts.

Typically, Leptospira are aerobic and motile spiroche-

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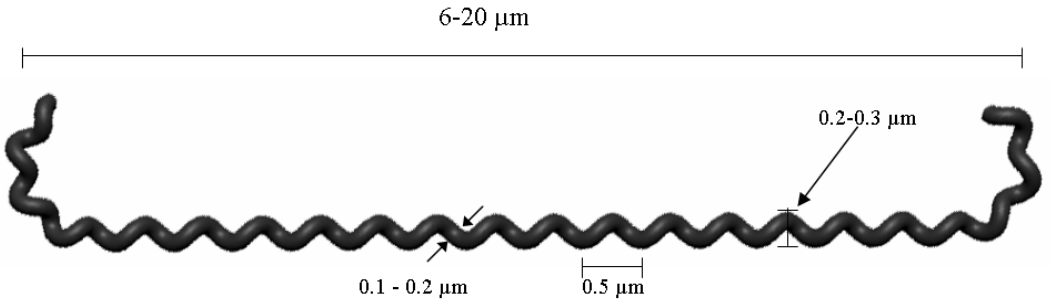

Figure 1. Side view schematic representation of the structure of *Leptospira*.

tes with helical or spiral structure and a unique (among the spirochetes) hook at both ends (Figure 1). They are about 0.1-0.2 µm wide and 6-20 µm long and have helical amplitude of approx 0.1-0.15 µm and a wave length about 0.5 µm (Faine et al., 1999; Goldstein et al., 1990). Their ultra-structure comprises a double cytoplasmic membrane, in close contact with a peptidoglycan layer and an outer membrane (Haake, 2000). The composition of the outer membrane of lipopolysaccharide (LPS) is similar to other gram-negative bacteria (Vinh et al., 1986), but it has lower endotoxic activity (Shimizu et al., 1987). Optimal conditions for the growth of this organism are well documented (Faine, 1982; Faine, 1999). The most suitable conditions for its survival outside the host are a moist environment with neutral pH and a temperature range between 20-32°C.

Leptospira are highly susceptible to adverse environments such as dry atmosphere, chemicals (chlorine or iodine in detergents), unfavorable pH (>8.0 or <6.5), magnetic fields (Triampo et al., 2004) and high temperatures (above 40°C). Ultraviolet radiation is part (5%) of the solar radiation impinging on the earth's surface every day. This radiation consists of UVA (Ultraviolet-A: 400-320 nm), UVB (Ultraviolet-B: 320-280 nm) and UVC (Ultraviolet-C: < 280 nm) in percentages of about 96, 3, and 1% of total UVR energy, respectively (Dissanayake et al., 1993). High-energy ultraviolet irradiation (wavelength peak at about 253.7 nm.) is effective in destroying leptospira and other microorganisms (Srikanth, 1995; Stamm et al., 1988).

Earlier studies have researched the effects of UVA on eukaryotic cells, animal tissues, and prokaryotic cells such as *Escherichia coli* (Pizarro, 1995), as well as other bacilli or coci bacteria (Taber et al., 1978). However, results found in other bacteria may not be able to be used to explain UVA's effects on spirochetes, due to differences in their morphological characteristics, motility and virulence-related structure. In addition, differrences also exist at the genomic and proteinomic levels.

For instance, Leptospira have two circular chromosomes, in contrast to the linear chromosomes and chromatids in Berrela. Also, unusual features are found in their ribosomal gene organizations. Within the genomic decoding of Leptospira, enormous diversity has been found in the genetic organization of different serovars. Thus, spirochetes could be considered a unique group in the microbial world.

In the present study, the effects of UVA stress on the growth and activity of *Leptospira interrogans* serovar Canicola was studied. Cell growth and viability have been determined by conventional microscopic agglutination test (MAT), dark-field microscopy, and spectrophotometry. Changes in antigens and protein expression in the cells have been determined by SDS-PAGE and immunoblot. This work is aimed to gain a better understanding of the response of leptospira under the stress of UVA irradiation, in order to develop effective strategies to limit their survival in natural environments by introducing a UVA light source or adapting the risk area to be more exposed to sunlight. Within this regard, various water treatment technologies (including direct photolysis and bacterial inactivation by inherently produced free radicals by UVA) could be improved greatly and economically.

MATERIALS AND METHODS

Strain and culture conditions of leptospira

Pathogenic *L. interrogans* serovar Canicola were obtained from the National Leptospirosis Reference Center, National Institute of Health (NIH), Thailand and grown in the Ellinghausen and McCullough liquid medium as modified by Johnson and Harris (EMJH) (Ellinghausen et al., 1965; Faine et al., 1999) at about 27°C. The samples were sub-cultured on a weekly interval. Each of the 3 ml bacterial cultures in a 1.5 cm diameter glass tube had an initial optical density (OD) at 400 nm of about 0.10, as measured by a UV-VIS spectrophotometer. This was equivalent to the 1+ level, as graded using a dark-field microscope, which in turn indicated a cell concentration of ~10^8 cells/ml.

UVA irradiation source

UVA radiation was generated by a 20 W T12 fluorescent lamp, Backlight (Sylvania Co.). The lamp produced a continuous emission spectrum (320-400 nm) and a peak at 365 nm, measured by a HR2000CG-UV-NIR high-resolution composite-grating Spectrometer (Ocean Optics, Inc.). The UVB and UVC radiation were absorbed by the glass tube. The experimental leptospira samples were exposed to UVA radiation only at an intensity of about 1.3 mW/cm^2 at a distance of 20 cm from the light source, as measured by a 1830-C optical power meter with

detector and calibration module, model 818-UV/CM (Newport Corporation).

Irradiation procedure

Separate leptospira samples were exposed to UVA at a distance 20 cm from the source of radiation for 0, 5, 15, 30, 45, 60, 120, 180, and 360 min on day 1, and then for several more days (1 to 7 days). After exposure, all cultured samples were maintained for a further 7 days. On 7^{th} day post-treatment, all samples were taken for measurement of their viability and growth using a dark-field microscope (Axiloab Pol 2, Carl Zeizz Co.) mounted with a CCD camera. Micrographs were taken using AxioVision AC 4.1 software. Semi-quantitative techniques based on the microscopic agglutination test (MAT), the turbidity-based method, and the UV-VIS spectrometer was also employed. Protein or antigenic changes were determined by using SDS-PAGE. Experiments were repeated at three times under the same conditions with separated occasions.

Growth and cell survival

The techniques mentioned above were used to monitor the bacterial growth and survival as follows.

Qualitative analysis by dark-field microscopy

The growth and survival of treated leptospira were determined by looking at cell density, mobility (Silva et al., 2001) and morphology. Due to their very thin size and the poor uptake of conventional dyes, leptospira could not be observed by an ordinary light microscope, thus a dark-field microscopy (DFM) was used (WHO, 2003). In DFM, an oblique light beam was cast onto the leptospira (lying on a microscope slide), using a special condenser when the central illuminating light beam was interrupted. The leptospira could then be seen as silvery threads on a dark background. To assess the effect of UVA on growth and survival, samples were diluted 10-fold on 7^{th} day post-treatment and observed with a dark-field microscope.

Semi-quantitative analysis by microscopic agglutination test (MAT)

MAT is a standard serological technique used in the diagnosis of leptospirosis. It is used in combination with the enzyme-linked immunosorbent assay (ELISA). It is a reference test and is used to detect the presence of antibodies and to determine their titer. It can also be used to determine the relative growth of a bacteria colony. The method is simple and consists of mixing the test serum with a culture of leptospira and then evaluating the degree of agglutination using a dark-field microscope.

A reference-specific antiserum against *L. interogans* serovar Canicola was required for the test. Leptospira samples were mixed with 2-fold dilutions of antiserum (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400) and incubated for 2 h at room temperature. The agglutination reactions were then observed under a 200x dark-field microscope and scored as follows: 4+, 3+, 2+, 1+ = 100%, 75%, 50%, 25% absence of free leptospira from the field, respectively.

Quantitative analysis by UV-VIS spectroscopy

An UV-VIS spectrometer (V-530 UV/VIS spectrometer, Jasco Internationl Co., Ltd.) was used to quantify the amount of Leptospira present by light absorption measurement in the UV-VIS spectrum (Faine et al., 1999; Perkampus, 1992; Tchamedeu et al., 2002). These were done on exposed leptospira cells (for different exposure times) taken on the 7^{th} day of their cultivation. Solutions of the leptospira cells were placed into cuvettes made with quartz SUPRASIL (200-2500 nm) with a light path of 10 mm (type no. 100.600-QG, Hellma Co.). A single beam spectrometer operating in the range of 200-800 nm was used. The OD or absorbency was taken at 400 nm (Tchamedeu et al., 2002). All samples were obtained with already used EMJH liquid medium as the blank.

Atomic Force Microscopy (AFM)

Scanning probe microscopy (SPM) (Digital Instruments Veeco Metrology Group, NY, USA) was used for AFM surface morphology imaging. Images were acquired in the contact mode showing height contours that highlight the spiral shape and fine surface morphology of leptospira. An AFM scanner with a hardware correction for nonlinearities of the piezoelectric element was used. The scanner has a maximum xy range of 125 by 125 µm and a Z range of 6 µm. Cantilevers measuring Si_3N_4, 125 µm long and 35 µm wide with a spring constant of 0.58 Nm^{-1} were used. To locate the area of interest in the samples and identify any bacteria, we used a built-in long-range on-axis microscope, capable of 5:1 zoom and x3500 magnification. Imaging was carried out at scan speeds between 1 and 50 µm/s. Images were acquired at 256 x 256 pixels. A typical imaging session began using a built-in optical microscope and by moving the x-y table to search for bacterial cells. The AFM cantilever was then moved forward to the surface close to the chosen bacterial cell. Each sample was prepared using the method described above. It was then dropped on a microscope glass slide and air-dried.

Scanning electron microscopy (SEM)

Samples were washed three times with normal saline (0.9% NaCl) at 10,000 × g for 10 min and dropped on a poly-L-lysine-coated cover glass slide for 1 h. Samples were prefixed directly with 2.5% glutaraldehyde for 2 h at room temperature and then washed three times with a 0.01 M phosphate buffer (pH 7.3) for 10 min. All samples were post-fixed with 0.1% osmium tetroxide. After fixation, samples were dehydrated with increasing concentrations of ethanol, then critical point-dried (HPC-2 critical point dryer, Hitachi) and coated with a platinum-palladium ion sputter (E102 ion sputter, Hitachi) for 2 min. The micrographs were taken with an accelerating voltage of 15 kV SEM (S2500, Hitachi) on the negative film.

SDS-PAGE and immunoblot

Whole cell solublization: Leptospiral cultures were centrifuged at 10, 000 × g for 10 min. The pellets were washed three times in normal saline (0.9% NaCl) and then solublized in a SDS-PAGE sample buffer composed of 0.35 M Tris.Cl (pH 6.8), 10% SDS, 30% glycerol, 9.3% dithiothreitol (DTT) and 0.175 mM bromophenol blue (Pope and Johnson, 1991).

Gel electrophoresis and immunoblot: For one-dimensional SDS-PAGE, other samples were solubilized in a SDS-PAGE sample buffer. The samples were heated at 100°C for 10 min (Pope and Johnson, 1991). Then 3 µl of each sample was loaded onto 12% polyacrylamide gels (Laemmli, 1970) and electrophoresed at 200 voltages for 55 min. The molecular mass protein standards (Amersham) used were rabbit muscle phosphorylase B (97 kDa), bovine serum albumin (66 kDa), hen egg-white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (20 kDa) and hen egg-white lysozyme

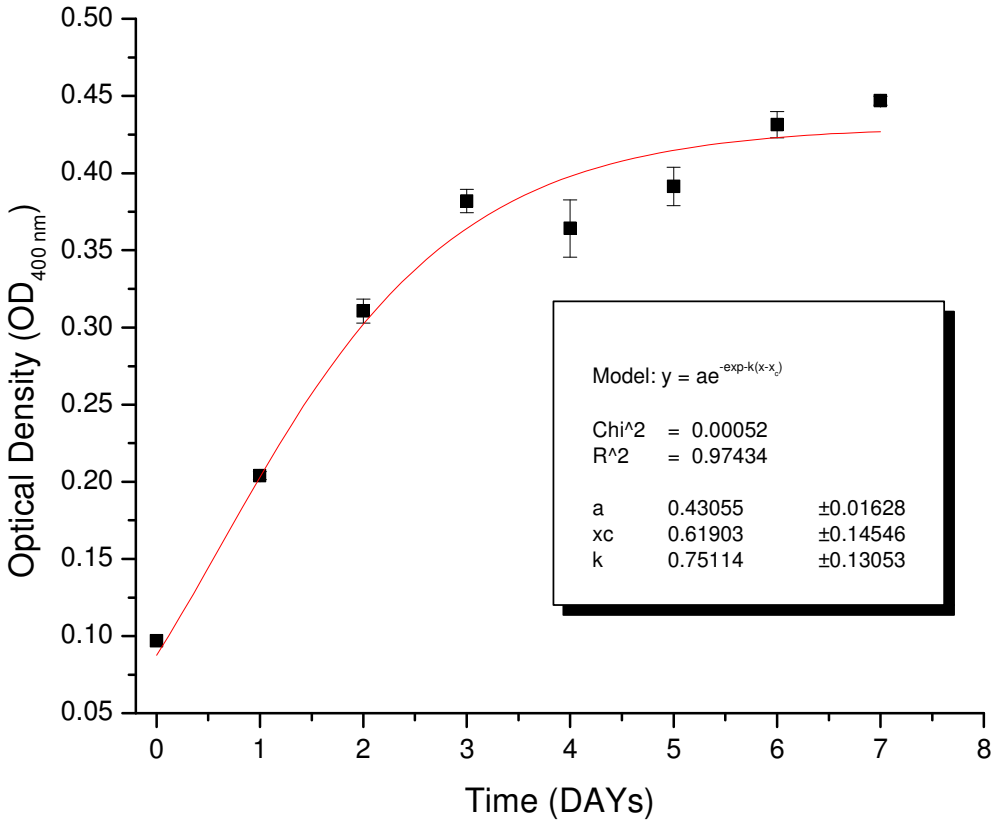

Figure 2. The growth curve of *L. interrogans* serovar Canicola. Relationship between Optical density (OD) values (at wavelength 400 nm) versus days of bacterial cultivation (1, 2, 3, 4, 5, 6 and 7 days) with EMJH liquid medium as a blank can be well fitted with Gompertz function.

(14 kDa). After electrophoresis, the gel was separated into two parts. One part was stained with Coomasie brilliant blue (0.025% Coomassie R-250, 40% methanol, 7% acetic acid) for 1 h and then destained with destaining solution I (40% methanol, 7% acetic acid) for 1 h and fixed with destaining solution II (5% methanol, 7% acetic acid). The other part was blotted onto a polyvinylidene difluoride (PVDF) membrane at 1.6 mA/cm^2 for 70 min. After transfer, membranes were incubated with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid (Merck) and washed with 2% skimmed milk and 0.2% Tween-20 (Sigma, USA) in phosphate buffer saline (PBS).

For immunological detection, the membrane was incubated for 1 h with primary antibody (rabbit reference antiserum specific to *L. interrogans* serovar Canicola) at 1:1,000 dilutions in 2% skimmed milk and 0.2% Tween-20 in PBS, and then washed three times for 10 min each in 2% skim milk and 0.2% Tween-20 in PBS. Thereafter, the membrane was transferred to a solution of secondary antibody [polyclonal goat anti rabbit immunoglobulin HPR (DakoCytomation P0448)] at 1: 2,000 in 2% skimmed milk and 0.2% Tween-20 in PBS, incubated for 1 h and then washed three times for 5 min each in PBS. Color was developed with a solution of 1.25 mg diaminobenzidine (DAB) and 5 µl 35% H_2O_2 in 10 ml of PBS. The membrane was then rinsed in several changes of PBS to stop the reaction, air-dried and photographed.

RESULTS AND DISCUSSION

The growth curve of *L. interrogans* serovar Canicola is given as a plot of the number of leptospira (proportional to the measured OD values) present at a particular time. This growth could be well-fitted to Gompertz function (Gompertz, 1825) ($y = ae^{-\exp -k(t-t_c)}$) with the parameters a = 0.43 ± 0.02, k = 0.75 ± 0.13, and t_c = 0.62 ± 0.15. The OD value which was proportional to cell number density increased exponentially to 400% (OD = 0.40) of the initial value after 3 days of cultivation. It then approached a steady state (OD of about 0.45 times of its initial value) until day 7 (last day of observation), as seen in Figure 2; this figure shows the Gompertz-like growth, which might be due to food limitation and increased cell toxicity due to over-crowding of the population. The presence of an intermediate region in the Gompertz curve indicates that in nature, bacterial life alternates between growth and stationary phases, primarily due to fluctuations in nutrient availability.

To see the effects of UVA irradiation exposure on *L. interrogans* serovar Canicola, all samples (control and treated) were cultivated for the same length of time (7 days). To gain a visual impression of what was occurring, the growth of the leptospira samples exposed to UVA radiation for 0, 30, 60 and 360 min (and for 1 to 5 days) was monitored using a dark-field microscope. Figure 3A shows the dark-field image samples cultivated for 7 days. The images clearly show a decrease in the number of bacteria present as the exposure dura-

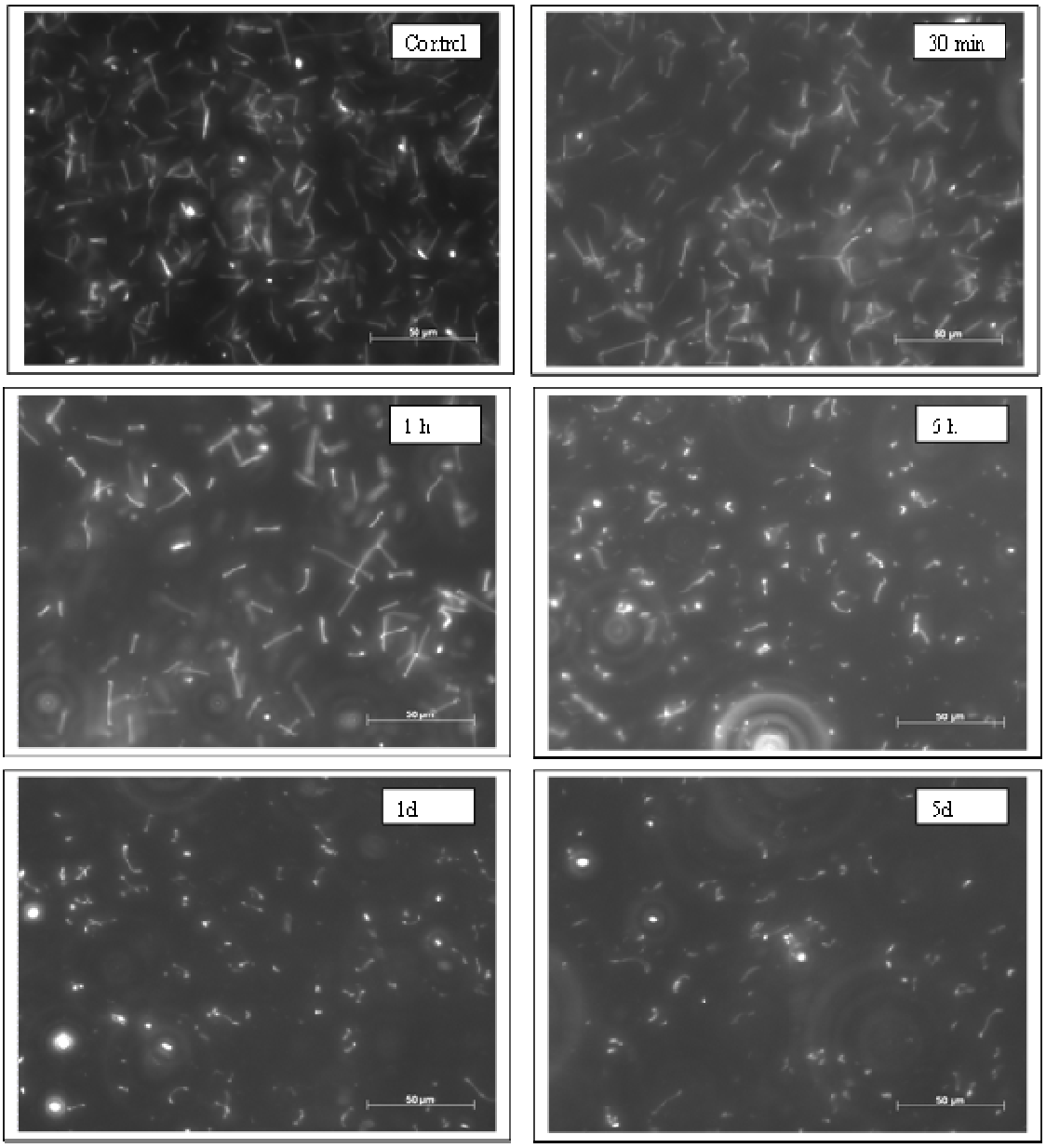

Figure 3A. Dark field micrographs of *L. interrogans* serovar Canicola exposed to UVA for different durations: 0 min, 30 min, 1 h, 6 h, 1 day, and 5 days. Images were taken at the 7th day of each experimental culture sample (diluted 1:10) with magnification 400x. Bars: 50 µm

tion increased.

The mobility of cells appeared also to decrease as the exposure time increased. Some untypical morphology was was observed after 1-day exposure. We used an atomic force microscopy (AFM) operating in the tapping mode to obtain images of the control cell and that which was exposed to the UVA for 1 day. Figure 3B shows that the spiral period had become longer. The SEM micrograph also exhibited the same trend as observed in Figure 3C. However, the mechanism by which the leptospira morphology changes is still unknown.

The OD of the leptospira exposed to UVA for various time durations is shown in Figure 4. Results show a decrease in OD, as the exposure time was increased up to 1 day (Figure 4A). For a larger time scale (day), the data indicates sharp decreases in the first day; then it more or less levels off as time goes on. However, one may argue that the OD slightly increases after one day (<0.05). This turbidity change could be due to an effect of the UVA, one that causes more fragmentation of the remains and results in increasing amounts of smaller debris or a cross section surface area.

These results were consistent with those obtained with conventional MAT measurements (Table 1). The agglutination reactivities of the bacteria exhibited a relative decrease as the exposure time increased. The

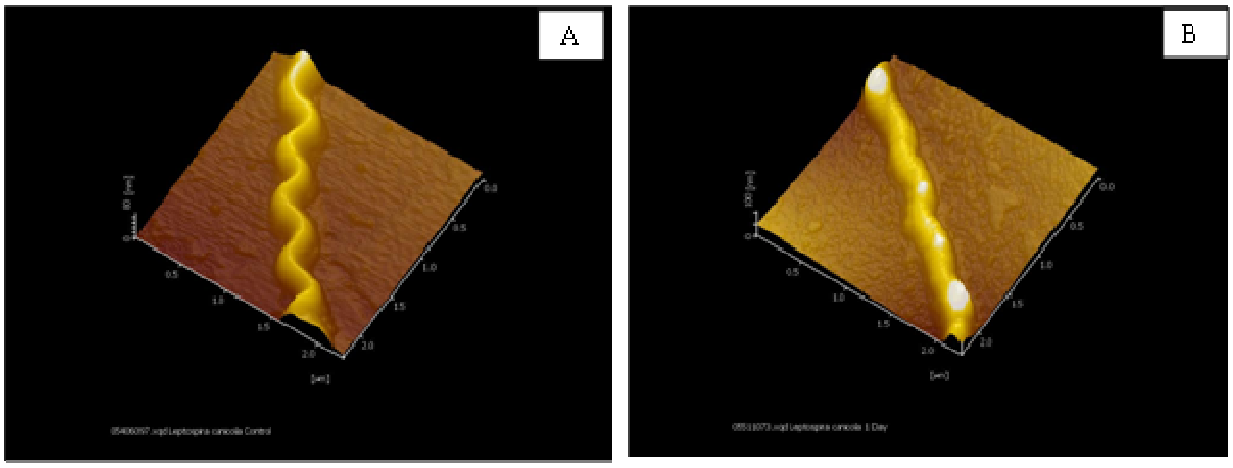

Figure 3B. AFM micrographs of spiral shape using atomic force microscope (Seiko Instrument Inc., Japan) in dynamic mode with micro cantilever type: SI-DF20 K-A102001604, f = 135kHz, spring constant 13 N/m (Seiko Instrument Inc., Japan). Scan area size was 2 x 2 μm^2. In the control sample unexposed to UVA, the leptospira had an approximate wavelength of 0.5 µm (A), but the 24 h UVA-exposed leptospira (B) had some cells with a longer period than others.

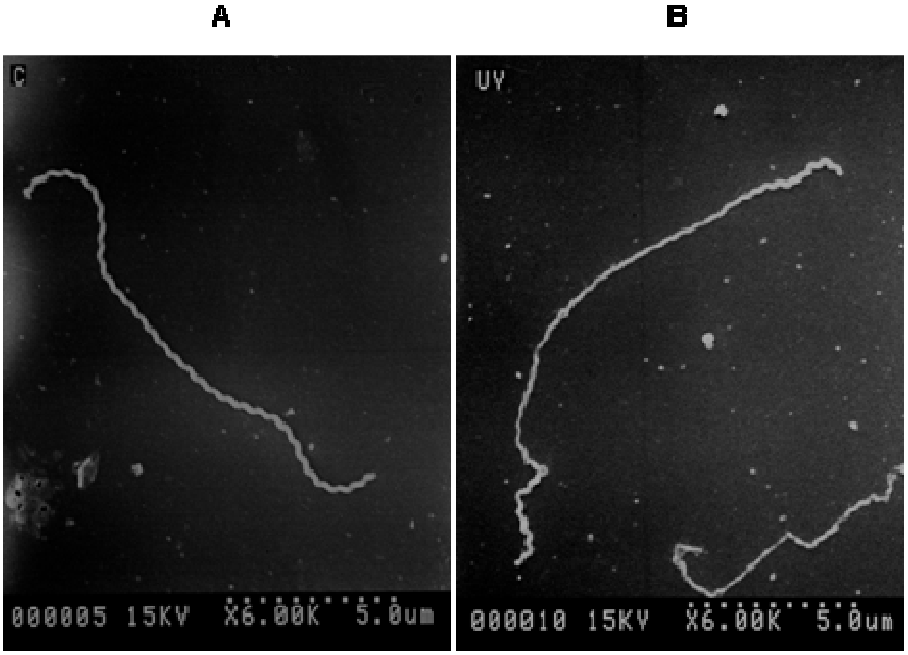

Figure 3C. SEM micrographs of spiral shape taken using scanning electron microscope (Hitachi, Japan) with 15 kV [Magnification 6000x. In the control sample unexposed to UVA, the leptospira had a consistent wavelength (A) compared to the 24h UVA-exposed leptospira (B), which had some cells with a longer period than others].

changes in agglutination reactivities became very small after being exposed to UVA for more than 1 day. Figure 5 shows the dark-field micrographs of agglutination of leptospira after reacting with specific antiserum (at dilution 1:100). It showed small changes in the agglutinating size and in the features of the exposed samples (Figure 5B) compared to those of the control cell (Figure 5A). A change in agglutination pattern was observed only after the samples were exposed to UVA radiation for at least 1 day.

To test the hypothesis that the effects of UVA irradiation up to 24 h (112.3 J/cm^2) may mainly cause temporarily injured or dead cells due to the UVA treatment, we performed the post treatment effect, or long-term effect, study. To do so, we re-subcultured the unexposed and exposed samples and monitored their growth in the

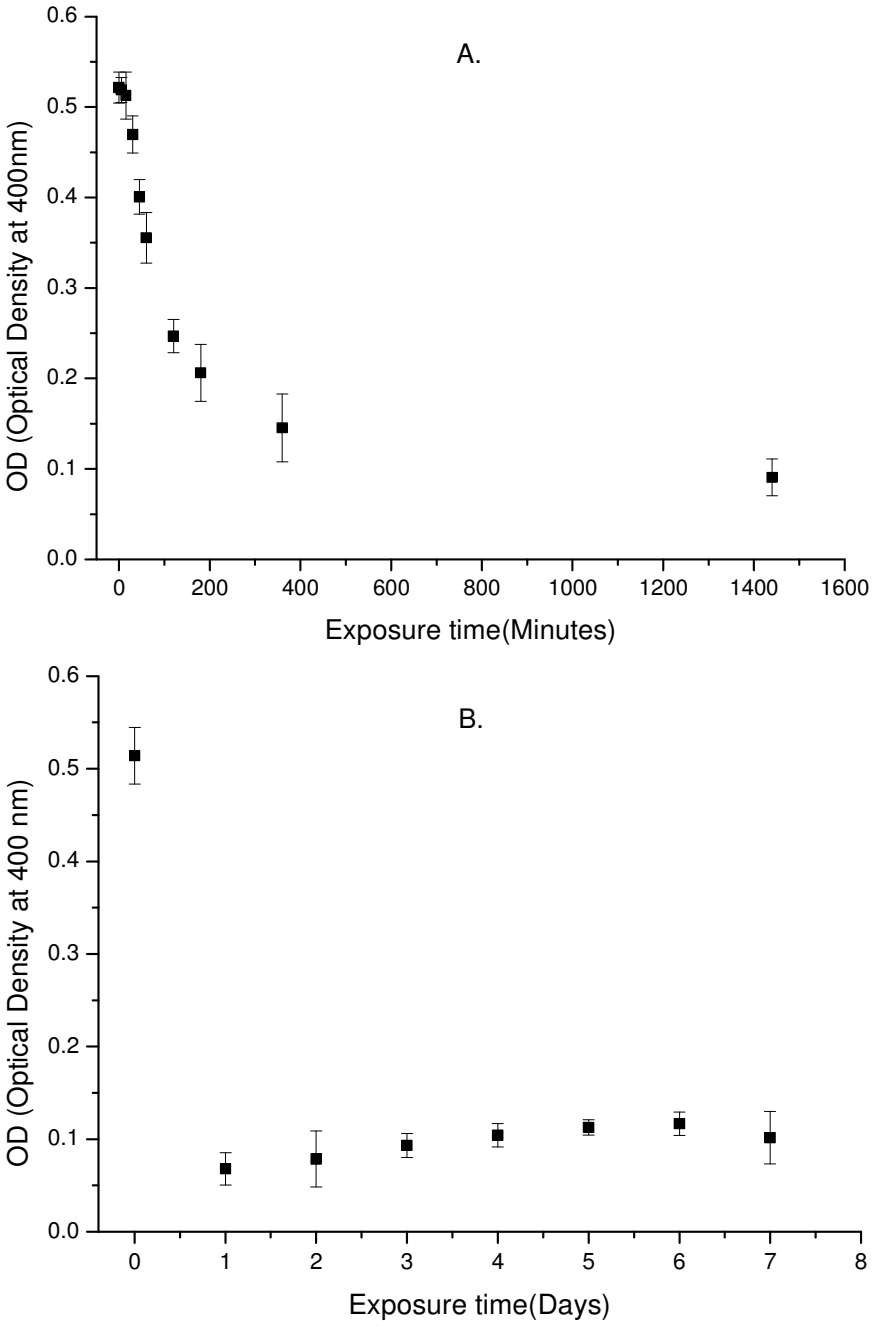

Figure 4. Dependence of the optical density ($OD_{400\ nm}$) of leptospira on exposure times. Exposure times (A) 0, 5, 15, 30, 45, 60, 120,180, 360, and 1440 min; and (B) 0, 1, 2, 3, 4, 5, 6, and 7 days. All samples were measured at the 7th day of cultivation and the experiment was repeated at 3 times.

dark. After 7 days of culture, the samples exposed to UVA for 24 h or more did not normally grow, that is, the pre-exposed cells were more or less dead (data not shown)—as these cells would have re-grown if they were just temporary injured or inactive. Although the cells exposed for less than 24 h could grow, their growth was not as good as the control cell. These results may imply that after treatment the cells were injured when exposed for less than 24 h and became more injured and/or killed after 24 h of UVA exposure.

SDS-PAGE and immunoblot tests were used to investigate UVA-induced antigenic denaturation. Electroseparated protein components were visualized on the gel by Coomasie blue staining, or transferred to PVDF membrane and immunostained with reference antiserum, specific to Canicola. The results of immuno-

Table 1. MAT reactivity of leptospiral samples after UVA exposure for various time intervals.

No.	Exposure time	Dilution of reference antiserum tested						
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
1	0 min	4+	4+	3+	2+	1+	1+	-
2	5 min	3+	3+	2+	2+	1+	-	-
3	15 min	3+	3+	2+	2+	1+	-	-
4	30 min	3+	3+	2+	1+	1+	-	-
5	60 min	3+	3+	2+	2+	1+	-	
6	120 min	3+	3+	2+	2+	1+	-	
7	360 min	2+	2+	2+	1+	-		
8	1 day	2+	1+	1+	-			
9	2 days	2+	1+	-				
10	3 days	2+	1+	-				
11	4 days	2+	1+	-				
12	5 days	2+	1+	-				
13	6 days	2+	1+	-				
14	7 days	2+	1+	-				

4+, 3+, 2+, 1+ = variable degree of agglutination reactivity.
- = did not agglutinate.

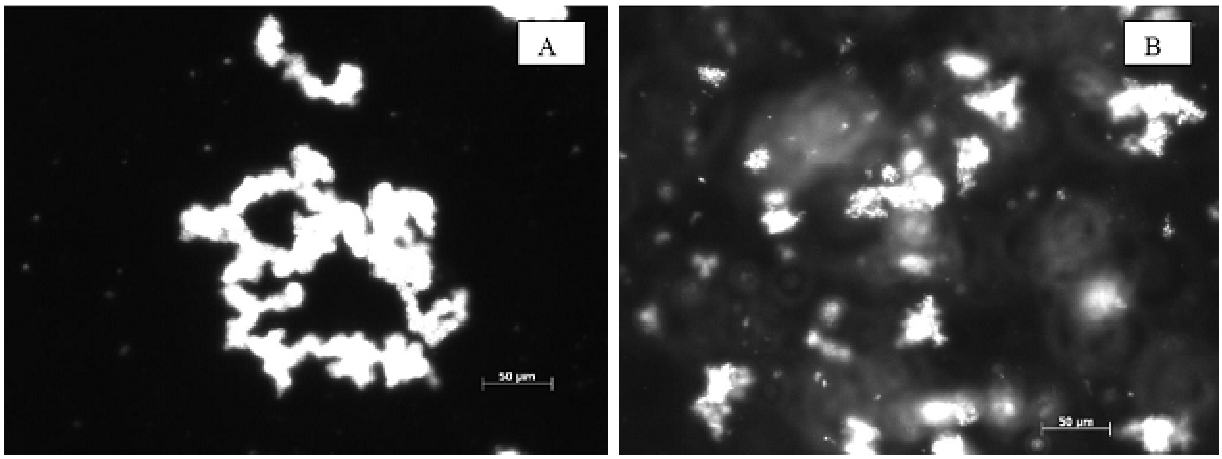

Figure 5. Dark field micrographs of agglutinated bacterial cells. The images were taken after reacting agglutinated bacterial cells with homologous antiserum (anti-canicola). (A) Micrograph showing complete agglutination of control or unexposed sample; (B) Micrograph shows incomplete agglutination of 1-day exposure sample at 1:100 dilution, with magnification of 200x.

blot for the unexposed (control) sample and UVA-exposed samples at 2, 6 and 24 h are shown in Figure 6. The sample exposed for 24 h (lane 4) showed the absence of antigen with a molecular mass of about 21 kDa—whereas this band was observed in the control (lane 1), at 2 h (lane 2) and 6 h (lane 3) the exposed samples and any other antigen components did not show effects from UVA exposure (data not shown).

Figure 7 shows SDS-PAGE analysis of the proteins in leptospira cells exposed to UVA radiation for 2, 6 and 24h. Compared to the control (lane 4), at 24 h exposure (lane 3) a 76 kDa protein band appeared. The 76 kDa band which appeared after 2 h exposure begins to disappear as longer radiation times are used (compare lane 1 with lanes 2 and 3). Proteins having molecular masses between 56 kDa and 70 kDa also appeared in lanes 2 and 3, possibly due to the degradation of 76 kDa protein, induced after 2 h of exposure to UVA.

The results of less than 24 h UVA exposure demonstrate that UVA could temporally cause cell damage or injures as a consequence of inhibiting the growth of leptospira. Hence, it allows growth recovery and subsequent re-growth of bacteria after UVA is removed. This was confirmed by the post-treatment results or the long-term effects study. In contrast, when the exposure time was longer (from 24 h up to 7 d), the permanent inactivation, or bactericidal effect, was consistently observed and once again can be confirmed by post-

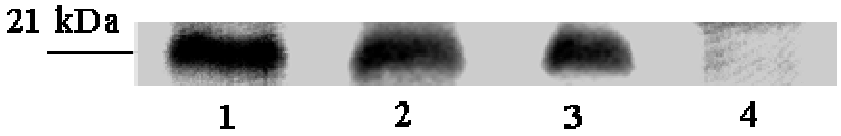

Figure 6. Immunoblot of *L. interrogans* serovar Canicola antigen. Antigens were extracted from the cells on the 7th day of cultivation. Lane 1: control without UVA exposure. Lanes 2-4: samples exposed to UVA for 2, 6 and 24 h respectively. The 21 kDa band disappeared after 24 h of exposure.

treatment studies. Therefore, these findings indicate that the UVA effects are dose-dependent.

Regarding this conclusion, our experimental set-up design may correspond to daily doses in real world environments. The UVA doses used here are consistent with those used in ref. Pfeifer et al. (2005). There, Maneewan and co-workers (Maneewan et al., 2004) measured the average daily intensity of solar radiation in Thailand to be about 17.5 MJ m^{-2} day^{-1} or 20.3 mW/cm^2. However, the UVA only accounts for about 5% of solar intensity or is equivalent to 1.03 mW/cm^2. Hence in terms of doses used, it may be reasonable to say that our experimental design is practical. However, there are other factors that may play roles in these effects, such as the depth of the water reservoir, the flow and the aeration rate of water, etc. Therefore, more work needs to be done to make connections between our findings and the possible future implementation of the leptospirosis control.

With regards to the change in morphology of UVA exposed leptospira compared with unexposed leptospira, an unusual thinner size and less spiral shape were remarkably observed. Possible explanations may involve the structure of the leptospira affected by UVA irradiation. This was very pronounced for the 24 h exposure treatment (Figure 3B and C). It is known that axial filament is the skeleton of the spiral structure (Bromley et al., 1979; Goldstein et al., 1996). Hence, it is reasonable to say that the changes in morphology due to UVA exposure might involve this structure. Specifically, the axial filament is responsible for the locomotion of leptospira (Brandling-Bennett et al., 1996; Faine et al., 1999; Nauman et al., 1969) damage to it thus could cause the leptospira to become less active and less mobile (observed under dark field OM observation).

Previously, Silva and co-workers (Silva et al., 2001) reported about the loss of helical shape due to axial filament damage. They demonstrated that the abnormal spiral shape of the leptospira could be subjected to pressure. Moreover, they also suggested that the leptospira could become less mobile as a result of morphological changes. These findings appeared to support our results.

The change in the formation pattern could possibly be due to low leptospira concentration or the denaturing effect of the antigen-antibody reaction. Therefore, we compared agglutination reactivities of low concentration in an unexposed leptospira sample to a sample exposed for 1 day that had produced the same OD and found the two patterns more or less qualitatively similar (data not shown). It was concluded that the changes in agglutination reactivities and reactivity patterns were due to the use of low concentrations of cells.

Now, we turn to the UVA effects on leptospira at the molecular level. It is known that UVA radiation induces cellular and molecular changes, such as damage to DNA, proteins and lipids in human and bacterial cells (Cadet et al., 2005; Pfeifer et al., 2005; Vile et al., 1995). They can induce the formation of reactive oxygen species (ROS) in the cells (Kramer et al., 1987; Merwald et al., 2005; Zhang et al., 1997), which may cause photo-oxidation of membrane-bound content and damage the cell membrane and DNA (Cheng et al., 2002; Ramabhadran et al., 1976). This may result in the lowering of growth and an associated denaturation of morphology. As spirochetes, including *L. interrogans*, have shown differences in their cell membranes and axial filaments from other bacteria, further studies on UVA exposure are required to gain insight into the mechanisms of this specific system.

One of the interesting observations made during the SDS-PAGE analysis was the appearance or induction of the 76 kDa protein (not found in control or unexposed cells) in *L. interrogens* serovar Canicola, after exposure to UVA radiation for 2 h. Nally and co-workers (Nally et al., 2001) reported that *L. interrogans* serovar Pomona could respond to external stress by synthesizing the proteins or other cellular constituents necessary for its survival in a changed environment, including temperature and pH. These findings may suggest that *L. interrogans* could respond to perturbed stress, including UVA stimuli, by expressing proteins. Guerreiro and co-workers studied leptospiral proteins recognized during the humoral immune response to leptospirosis in humans, focusing on *L. interrogans* serovar Copenhageni (Guerreiro et al., 2001). They reported that two major antigens, p62 and p76 (identified as chaperones GroEL and DnaK respectively), were found in these bacteria in the 2-D immonoblot of the proteins, when probed with pooled convalescent-phase sera from leptospirosis patients.

The p76 protein (with a band under 82 kDa) is a bacterial heat-shock protein and is upregulated by elevated temperatures. It was identified as the target of a humoral response during natural infection, and it

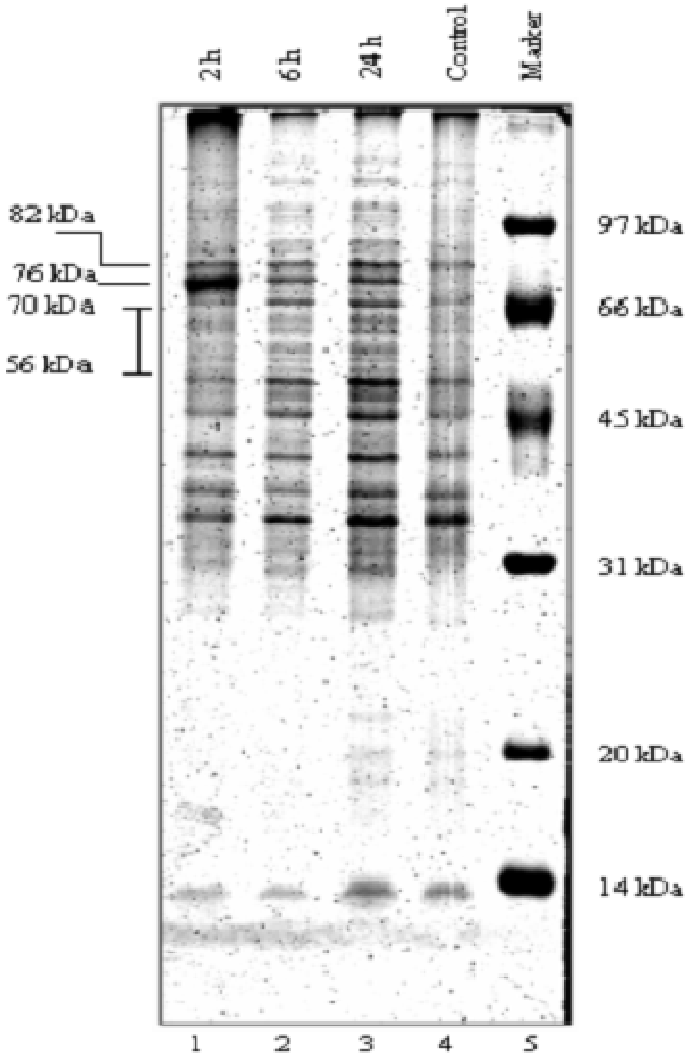

Figure 7. SDS-PAGE analysis of the protein expression in leptospira. The gels were stained by Coommasie brilliant blue staining in presence of UVA for 2 h (lane 1), 6 h (lane 2), 24 h (lane 3), control sample (lane 4), and a standard molecular mass marker (lane 5). All samples exposed to the UVA radiation expressed the 76 kDa band significantly.

plays an important role in the repair, folding and assembly of proteins following heat stress (Zeilstra-Ryalls et al., 1991). *L. interrogans* serovar Hardjo could also rapidly synthesize p62 and p76 proteins, as a consequence of a sudden increase in temperature (Stamm et al., 1991).

From our results, SDS-PAGE analysis showed that the intensity of the 76 kDa band of 2 h exposed cells was very noticeable when compared with samples exposed for 6 h or longer. To understand these results, it may be useful to relate them to growth. The growth curves show that compared with 2 h or longer exposed samples, the 2 h exposed group had a much greater number of leptospira, including those which were active and alive. These active leptospira are the ones that are believed to be able to express stress-response proteins.

Concerning our electrophoresis results, this interesting phenomenon may suggest that only active cells express 76 kDa proteins for responding to environment stress. Hence, this is why the 76 kDa protein's appearance faded as exposure times (6 h or higher) became longer. From, these findings, it might suggest that if we want to treat or get rid of leptospira, the dose to be used must be large enough to do the job. Otherwise, leptospira may just temporarily be inactive, or somehow induce a defense mechanism, or respond to treatment.

Another interesting finding was the disappearance of the 21 kDa protein band in the immunoblot of *L. interrogans* cells that were exposed to 24 h of UVA radiation. This result may indicate denaturation of this antigenic component at high dosages of UVA. Previously, Cullen and coworkers (Cullen et al., 2002; Cullen et al., 2003) studied *L. interrogans* serovar Lai and reported that a 21 kDa protein (lipL21) was found to

be the second major constituent of the outer membrane proteome. It was also found later that LipL21 is expressed during infection and conserved in the pathogenic Leptospira species, whereas it was not detected in nonpathogenic Leptospira biflexa (Priya et al., 2003).

Therefore, this evidence seems to indicate that the degradation of the 21 kDa band due to UVA might reduce the virulence of *L. interrogans*, which are pathogenic leptospira. However, more work needs to be done to draw any conclusion about the present study in terms of the correlation between the 21 kDa band of the UVA exposed sample and leptospira virulence.

The above findings may explain why leptospirosis mainly occurs in the rainy season. The water-borne bacterial nature of leptospira and lesser UVA radiation exposure during the rainy season may result in optimal conditions for the growth of leptospira. Of course, water and tropical climate-like environmental conditions are certainly major factors contributing to the growth and spread of these leptospira, UVA from sunlight also could contribute to the spread of leptospirosis. However, it should also be mentioned that based on our study, UVA radiation may be used as a bactericidal or bacteriostatic factor to further develop new strategies to control leptospira in the environment.

For example, one could adapt or set the landscape of households and environments which are susceptible (or at very high risk) to more UVA radiation exposure. A combination of UVA radiation with other photocatalytic materials like titanium dioxide (TiO_2) may be another alternative (Fujishima and Honda, 1972; Ireland et al., 1993).

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